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# (54) Protein product

(57) A process for the production of soluble collagen from natural collagen-containing material, such as hides or ossein, which process comprises treating the material with a culture from the group of lactic acid bacteria or a purified enzyme from such a culture, in an acid medium, and then dissolving the product in an acid solution.

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SPECIFICATION

**Protein product**

The present invention relates to the production of collagen from natural materials. More particularly, it relates to the production of soluble collagen for use as finings in the clarification of fermented liquors, the collagen being derived from natural products, especially those of a mammalian nature.

Isinglass is nearly 100% protein, substantially of a collagenous nature. The use of Isinglass as a clarifying agent has been known since antiquity. It has been particularly used for clarifying fermented liquors, especially beer (and wine). Isinglass has been traditionally made from fish swim bladders.

In the production of Isinglass in the traditional manner, the swim bladders are excized from the fish, collected, dried, physically treated to remove unwanted matter and impurities, further cleaned as necessary (sometimes including the use of hydrogen peroxide) and divided, for example by making into strips, shredding or the like. The material is then "cut" with an acid usually an organic acid (for example tartaric, citric, lactic or acetic acid) in an amount which preferably produces a pH in the "cutting" vessel of about 2.5 to 3.0. The amount of acid used is usually about 10% to 15% based on the weight of the dried swim bladder. Less acid can be employed, or even more (for example 25%) to reduce the "cutting" time. A preservative is also employed and it is usually sulphur dioxide in the form of sulphurous acid or a sulphite, preferably being added progressively at selected stages during the process. The resulting product is normally strained to remove extraneous (including "uncut") matter after dilution if desired. The product usually has a pH of 2.5 to 3.0 and an acidity (calculated as tartaric acid) of about 0.1% to 0.15%. The sulphur dioxide content of the product for use as finings in the clarification of fermented liquor, for example beer, is about 300 ppm.

Attempts have been made in the past to produce suitable substitutes for Isinglass for use as finings. Some mammalian materials have been investigated as starting materials for substitutes for Isinglass, such as bone and hide. Cattle hides were suggested as long ago as the 1930's. In the 1960's attempts were made to use eucollagen as finings. British Patent 990276 disclosed a fibrous sheet material consisting wholly or partly of fibrils regenerated from eucollagen. Eucollagen is a form of collagen, soluble in acidic media, obtained by treating raw material (skin, bone, ossein, sinew or the like) with alkali, in the presence of a salt, e.g. sodium sulphate, which protects the collagen from hydrolysis. Material obtained in this way can be dissolved in acidic buffer solutions and regenerated therefrom in fibrous form by a change of pH or ionic concentration or the addition of proteoglycans, tannin, or large molecules. The fibrils may be produced by regeneration in an aqueous slurry of fibres, which may be of tanned or untanned leather or of vegetable origin. The fibres are then formed into a sheet by filtering and drying. The materials so formed may be plasticized or tanned.

British Patent Specification 1184502 disclosed a composition for fining beer comprising an acid preparation of eucollagen. The eucollagen may be prepared by alkaline treatment of ossein or hide collagen at raised temperatures e.g. between 18°C and 29°C, in the presence of a substance capable of preventing swelling of the collagen, e.g., sodium sulphate. The acidic constituent of the preparation may be citric, lactic or tartaric acid. According to an example, dry ossein (decalcified bone) is soaked in sodium sulphate solution, the sulphate liquor is removed and used as the solvent for some sodium hydroxide, and this solution is added to the ossein and allowed to act for four days at about 20°C. At the end of this time the ossein is drained, washed in running water and acidified with hydrochloric acid to pH 4. The ossein is then washed again until it is almost neutral and it is then dissolved with agitation into 0.2% tartaric acid solution to give 10 litres of finings at pH 2.5. In other examples the collagen source is cow hide and tannery split pieces, respectively. Beer is treated with a solution of mixed ossein and hide eucollagens. Such a composition did not appear to find commercial applicability in the fining of beer.

At the beginning of the 1980's attempts were made to use natural bovine collagen as finings for beer and wine. One disclosure of such material indicated that a particular bovine raw material selected from ox hide of a specific age range was subjected to a sequence of washing, basification (under "further alkaline conditions"), neutralization and acidification steps, the acid swollen product being subjected to high shear.

Other disclosures of apparently the same product suggested that the ox hide of a specific age range was suggested that the ox hide of a specific age range was dehaired, limed and split to obtain the internal or corium layer. This layer was decalcified (with ammonium sulphate) and buffered to an acid pH. It was reduced to a controlled particle size (in the form of pellets) by mechanical grinding. The ground product was then subjected to a controlled alkaline swelling medium, dissolved in citric acid and allowed to stand. Presumably some form of neutrallization was also carried out between the swelling in the alkaline medium and the solution in citric acid. It would appear that the alkaline swelling medium comprised sodium sulphate and sodium hydroxide and was followed by washing with water.

It was reported that the product of such process had a concentration of 0.5% of collagen of which at least 60% was soluble collagen. The pH was reported to be 2.5 to 3.0, the intrinsic viscosity was over 17 dl/g at 15°C, and the molecular weight was 100% over 120000. The product for use as finings was reported to contain 500 ppm sulphur dioxide as preservative and to have a solids content of 1.5% to 2.5%. It would appear that such a product has not achieved practical and commercial success. As described above, in such a process the hide was pretreated with alkali prior to solution in the organic (citric) acid. Such treatment of hide in alkaline solution has been traditional in the art of the hide treatment to remove the hair and to "open up" the skin. A major part of the "opening up" process consists of removing proteoglycans such as hyaluronic acid and

chondroitin sulphate which help to stabilize the structure of the collageneous proteins. It is considered that the alkaline treatment modifies the protein by partly removing amine and amide groups.

We have now found that soluble collagen can be produced from natural material including mammalian derived material such as animal hide or skin by a process which does not use an alkaline pretreatment, but instead employs a culture medium of the lactobacillus type or the like.

According to the present invention there is provided a process for the production of soluble collagen from natural collagen-containing material which comprises treating the natural collagen-containing material with a culture from the group of lactic acid bacteria or a purified enzyme preparation from such a culture, in an acid medium, and then dissolving the product in an acid solution.

The present invention also provides a method of fining a liquid containing suspended solid particulate matter which comprises adding to the liquid a fining composition comprising soluble collagen produced in accordance with the process of the present invention. The liquid to be fined may be a fermented liquor such as, for example, an alcoholic beverage. Examples of such beverages which may be treated are beer and wine.

By means of the present invention, it is possible to produce soluble bovine collagen in substantially native state. Whilst the present invention will be particularly described with reference to the use of bovine hide/skin, it may be possible to use any animal material containing collagen, particularly hides and skins, bone and possibly tendons, derived from sheep, goats and other mammals, as well as skins of fish and fowl. In the particular application to materials of a bovine origin, one can use fresh or salted skin. Fresh calf skin, especially that of a very young animal which has no been exposed to biocides in particularly easy to employ.

The skin is preferably first or pre-treated to substantially remove the non-collagen part, for example, the flesh part and the hair part (and epidermis), followed by washing, usually with water. When using bovine hide, it is usually Green fleshed, i.e., adipose tissue removed by machine, and then washed. Soaking, for example up to four hours with occasional mechanical action, is preferably then undertaken.

Particularly useful cultures are of the family lactobacillae, especially lactobacillus plantarum. A purified enzyme preparation, obtained for example by salting out, may be employed.

Usually a Starter Culture of lactobacillus plantarum is prepared by infecting an appropriate solution and incubating. An example of an appropriate solution is a whey medium comprising, by weight, 0.1% sodium chloride, 0.1% potassium chloride, 0.05% sorbic acid, 0.02% manganese sulphate, 33% whey and 66.73% water, incubation being carried out at 30°C to 35°C and a pH of 3.5 to 4.5 for 24 hours.

It is highly desirable that the process be conducted using a comparatively small amount of lactobacillus plantarum culture to innoculate the dehairing medium to avoid damage to the skin.

The culture may be used in amounts up to 30 parts - with 270 parts of medium in a 300% float. While lactose can be used as the medium, it is preferable to use whey since it reduces attack on the collagen and maximizes proteoglycan removal. An example of a suitable float is a 300% float of 6 parts of lactobacillus plantarum culture with 294 parts of whey medium.

In the treatment with the culture, the material being treated, for example bovine (particularly calf) skin is immersed in the starter culture nutrient medium. Whilst times of up to 6 days or more may be used, often only 48 to 56 hours are needed. Indeed, it may only be necessary to use up to 48 hours and even only up to 24 hours in the case of light or very young skins. The pH employed is usually in the range of 2 to 6, preferably 3.5 to 4.5. It is useful to use lactic acid to lower the pH if necessary. Temperatures employed are usually 20°C to 45°C, preferably 30°C to 35°C.

Any mechanical action (agitation) during the dehairing in the lactobacillus plantarum-containing medium should be kept to a minimum to avoid damage to the skin.

After treatment with the culture, the treated material can be washed to remove non-collagenous matter, hairs and the remains of the culture medium. Such washing is usually performed with water, but a surfactant can also be present. Temperatures of less than 30°C, preferably less than 25°C and usually substantially ambient can be used. The non-collagenous matter removed at this stage can include such matter formed during the culture treatment step.

Control can be exercised by monitoring so as to avoid damage to the skin, which is first apparent as grain peel when hair removal is attempted. Hairs and/or epidermis are easily removed such as by brushing or pulling. Gentle mechanical action using a machine may be employed. If, however, dehairing is not complete by such simple mechanical means then short hair which is still present may be removed by oxidative action, for example using sodium chlorite and glycollic acid.

The product is then washed and may be bleached with hydrogen peroxide or like materials, for example sodium perborate.

Indeed it may be useful to treat, after the culture treatment step, with hydrogen peroxide. Such treatment can be conducted after the culture treatment step but before washing and hair removal and/or after said working and hair removal. Hydrogen peroxide stops the process conducted by the culture, bleaches the product, keeps the product sterile and generally improves it. Usually at least 0.5% of hydrogen peroxide is used.

The dehaired skin is preferably chilled and then split so as to provide a grain layer and also a corium layer which is richer in collagen. It is preferred, but not essential, to at least chill the skin to assist such splitting.

The dehaired products may be pretreated (before dissolution in the weak organic acid) by drying, and may be ground.

The product, preferably can be ground to a fine powder

(for example, to one passing through a 20 mesh screen). The powder may then be solvent degreased followed by low or ambient temperature solvent removal. Such degreasing, for example by dichloromethane, makes the product more acceptable for use.

The material, which may have been ground to a powder, is then dissolved in a suitable acid. The acid employed is desirably a weak acid, normally organic, for example, citric or tartaric acid. Whatever acid is employed, it is desirable to use one and an amount thereof which will yield solution of moderate pH. The pH employed is usually in the range of 2 to 4, preferably 2.5 to 2.7. In connection with the pH, the effect of sulphur dioxide which may be present (see hereinbelow) must be taken into account. The dissolution may be performed by extraction over a fairly long period. The temperature is desirably not above ambient temperature (usually about 25°C) but may be lower (for example 4°C). The higher the temperature, the lower the time required for dissolution. Times can be of the order of 24 to 56 hours. Using citric or tartaric acid, the amount of acid is usually 0.04 to 0.5% W/V.

Preferably sulphur dioxide, usually in an amount of about 500 ppm, is present as a preservative.

The extracts may be clarified by a process such as straining to give slightly cloudy viscous solutions of the collagen. While solutions prepared for use usually contain about 6 to 11 mg/ml soluble collagen, concentrates of strenths up to about double these amounts can be prepared. A 40% to 90% yield is usually obtained.

An example of a particular dissolution procedure is as follows:-

Degreased powder was added gradually to a stirred solution of 0.2% W/V citric or tartaric acid containing 500 ppm $SO_2$. This procedure minimised the clumping whch otherwise occurred. The suspension was agitated for 48 to 56 hours on a rotary shaker at 4°C. The flasks were turned end over end at approximately 60 rpm. but towards the end of the period the solutions became too viscous to move when rotated at this rate. The suspension was vacuum filtered through coarse cloth filter (net curtain of 300 meshes/$cm^2$ was found suitable) into a receiver cooled with ice.

The collagen was found to have an isoelectric point in the range pH 7.5 to 8.5. It was also found that only insignificant decomposition of the collagen occurred during the process. Negligible quantities of collagen are found to be solubilized not only in the optional pretreatment step, but also in the culture treatment step.

The collagen produced by such a process was compared with Isinglass in the fining of several different types of beer. Collagen samples from both the corium and grain layers were tested. Al of them achieved satisfactory clarification of the various beers, giving a compact deposit and crystal clear resulting beer, for example about 5.8 on a standard scale of 0 to 6.

The collagen produced by the process of the present invention is particularly useful for finings for use in the clarification of beer (and wine). However, it may also be used in a multitude of application for which collagent is already used. Examples of such use include cosmetic industry and for artificial skins, films, tubes, haemostatic sponges and flour for surgery, biodegradable threads, cell culture and films as supports for enzymes an other macromolecules.

The present invention will be further illustrated with reference to, but in no manner limited to, the following Examples.

*Example 1*

Calfskins, slaughtered at birth (either salted or fresh) were machine fleshed to remove the majority of the adipose tissue adhering to the flesh side of the skins. The skins were then soaked for four hours in a paddle using a 300% float of water and occasional mechanical action. The fleshed and washed skins were then drained and weighed.

An innoculum was prepared by innoculating 200 gms. of whey medium with a culture of lactobacillus plantarum and maintained at 35°C in an incubator for 24 hours.

On the basis of each calfskin weighting 2 to 3 kg., a 300% float of whey medium (i.e. about 9 kg) was prepared by warming 6 litres of water to 30°C and adding thereto:- 2.97 kg whey, 9 gms sodim chloride, 9 gms potassium chloride, 4.5 gms sorbic acid and 1.8 gms manganous sulphate. The pH was then adjusted (as necessary) to 4.5 using lactic acid.

The calfskins (fleshed and washed as described above) were then immersed without mechanical action at 30 to 32°C for a period between 48 and 56 hours in a solution comprised of 294% of the whey medium and 6% of the innoculum (based on the drained weight of the skins). The pH of the bath was checked regularly to ensure that it remained between 3.5 and 4.5. The skins were taken from the bath when tests showed that the hair could be removed from the skin by hand pulling.

The hair was then pulled from the skins and, after rinsing in water, the skins were cut into 9 inch by 6 inch rectangles. These were cooled to 4°C in a refrigerator and split with a Camoga 300S band knife splitting machine into a Grain and Corium split.

The splits were freeze dried for approximately 16 hours down to approximately 10% moisture and ground in a Wiley mill to pass 20 meshes per inch. The ground powder was extracted in a Soxlet extractor for 4 hours with dichloromethane. The degreased powder was spread out on trays and the majority of solvent evaporated off at ambient temperature. Final traces of solvent were removed under vacuum.

1.6 gms of citric acid were dissolved in 800 cm3 water and small quantities of a saturated solution of sulphur dioxide added to achieve a concentration of 500 ppm. Approximately 10 gms of the degreased powder were slowly stirred into this solution in a 1-litre bottle which was then agitated for 48 hours at 60 rpm

in a rotary shaker at 40°C.

The extremely viscous solutions were then filtered under vacuum through a 300 meshes per cm cloth filter when approximately 660 cm3 was recovered. The solutions contained between 1250 and 1575 ppm of total nitrogen.

The solutions were diluted with water to the required concentration for testing and stored in a refrigerator at 4°C until tests were carried out.

Table 1 shows the percentage of total nitrogen in the solutions obtained and all four solutions were diluted to a concentration of 840 ppm nitrogen before being compared with a sample of Isinglass prepared from fish swim bladders at the same concentration.

All five samples were added to two types of beer (A and B), well mixed and allowed to stand at 18°C. The rate of addition to the beers was 2 pints per barrel of 36 gallons (i.e. 0.694% V/V.)

The compaction of the sediment and the clarity of the supernatent liquor was assessed by an experienced quality control chemist of an Isinglass producer using a scale of 1 to 6. The results are also shown in Table 1.

*Table 1*

| *Sample* | *Nitrogen %* | *Assessment A* | *B* |
|---|---|---|---|
| Salted Corium | 1576 | 5.8 | 5.8 |
| Fresh Corium | 1662 | 5.8 | 5.8 |
| Salted Grain | 1257 | 5.8 | 5.8 |
| Fresh Grain | 1528 | 5.8 | 5.9 |
| Isinglass | - | 5.8 | 5.8 |

It can be seen that soluble collagen prepared in accordance with the present invention fines beer as effectively as Isinglass. More concentrated solutions are achieved with fresh skins rather than salted and the grain protein is at least as effective as the corium protein although it is a less pure form of collagen.

*Example 2*

Samples of freeze dried, ground and degreased material prepared as generally described in Example 1 from the corium split of fresh calf skin were dissolved in both tartaric and citric acid. The fining capability of these two collagen solutions was compared with Isinglass as in Example 1 using solutions of approximately 900 ppm total nitrogen used at the rate of 2 pints per barrel of 36 gallons on three beers (A, C and B). The results shown in TAble 2 were obtained after standing 20 hours.

*Table 2*

| *Sample Dissolved in* | *Beer B* | *Assessment Beer C* | *Beer D* |
|---|---|---|---|
| Citric acid | 5.5 | 5.8 | 4.5 |
| Tartaric acid | 5.8 | 5.8 | 5.0 |
| Isinglass | 5.8 | 5.8 | 4.8 |

CLAIMS

1. A process for the production of soluble collagen from natural collagen-containing material which comprises treating the natural collagen-containing material with a culture from the group of lactic acid bacteria or a purified enzyme from such a culture, in an acid medium, and then dissolving the product in an acid solution.
2. A process as claimed in claim 1, in which the natural collagen-containing material is bovine hide/skin.
3. A process as claimed in claim 2, in which the natural collagen-containing material is fresh calf skin.
4. A process as claimed in claim 3, in which the fresh calf skin is that of a very young animal which has not been exposed to biochemicals.
5. A process as claimed in any one of claims 1 to 3, in which the natural collagen-containing material is first treated to substantially remove the non-collagen part, followed by washing, prior to treatment with the culture.
6. A process as claimed in any of claims 1 to 5, in which a culture of lactobacillus plantarum is used.
7. A process as claimed in any of claims 1 to 6, in which whey is used as a nutrient medium.
8. A process as claimed in any of claims 1 to 7, in which any agitation of the material being treated with the culture is not so great as to cause substantial damage to the material.
9. A process as claimed in any of claims 1 to 8, in which, after treatment with the culture, the treated material is washed to remove non-collagenous matter, hairs and the remains of the culture medium.
10. A process as clai treatment with the culture, is

treated with hydrogen peroxide.

11. A process as claimed in any of claims 1 to 10, in which the dehaired material is split so as to provide a grain layer and a corium layer.

12. A process as claimed in any of claims 1 to 11, in which there is a degreasing step prior to dissolution of the product in an acid solution.

13. A process as claimed in any of claims 1 to 12, in which the acid is a weak organic acid.

14. A process as claimed in claim 13, in which the weak organic acid is citric or tartaric acid.

15. A process as claimed in any of claims 1 to 14, in which sulphur dioxide is present in the product dissolved in the weak acid.

16. A process for the production of soluble collagen substantially as hereinbefore particularly described.

17. A method of fining a liquid containing suspended particulate matter which comprises adding to the liquid a fining composition comprising soluble collagen produced by a method as claimed in any of claims 1 to 16.

18. A method as claimed in claim 14, in which the liquid is fermented liquors.

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